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Rational Design of Pathogen-Mimicking Amphiphilic Materials as Nanoadjuvants

SUBJECT AREAS:
IMMUNOLOGY
BIOMATERIALS
NANOPARTICLES
COMPUTATIONAL BIOLOGY

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An opportunity exists today for cross-cutting research utilizing advances in materials science, immunology, microbial pathogenesis, and computational analysis to effectively design the next generation of adjuvants and vaccines. This study integrates these advances into a bottom-up approach for the molecular design of nanoadjuvants capable of mimicking the immune response induced by a natural infection but without the toxic side effects. Biodegradable amphiphilic polyanhydrides possess the unique ability to mimic pathogens and pathogen associated molecular patterns with respect to persisting within and activating immune cells, respectively. The molecular properties responsible for the pathogen-mimicking abilities of these materials have been identified. The value of using polyanhydride nanovaccines was demonstrated by the induction of long-lived protection against a lethal challenge of *Yersinia pestis* following a single administration ten months earlier. This approach has the tantalizing potential to catalyze the development of next generation vaccines against diseases caused by emerging and re-emerging pathogens.

Over the centuries, control of infectious diseases has often been hampered by the use of suboptimal tools and a lack of knowledge to prevent the spread of epidemic and pandemic diseases, including a dearth of efficacious vaccines. A transdisciplinary approach based on a new paradigm of immune activation is needed to rationally design next generation adjuvants and vaccines. Typically, adjuvants fulfill three roles: 1) act as a depot; 2) direct antigen to antigen presenting cells (APCs); and 3) induce co-stimulatory signals on APCs necessary for activation of naïve T cells. Unfortunately, current adjuvants approved for human use are often based on off-the-shelf materials that were not originally intended for use as adjuvants and allow pathogens to evade host defenses. The next generation of efficacious vaccines must incorporate adjuvants that can be tailored to generate the optimal immune response that provides protection against the target pathogen. In this work, we describe a bottom-up approach to design “pathogen-mimicking” nanoparticle adjuvants that has provided new insights into the rational design of customized vaccine delivery vehicles to induce long-lived, protective immunity.

An ideal vaccine will mimic the way in which a naturally occurring infection induces a robust immune response yet avoid the undesirable effects of disease¹. Current approaches to enhance the efficacy of a vaccine with an adjuvant often are designed to signal the innate immune system through a limited set of germ-line encoded pattern-recognition receptors (PRRs). These receptors recognize a series of conserved pathogen-associated molecular patterns (PAMPs)². The design of non-toxic polymeric materials that activate APCs without deleterious side effects will lead to efficacious vaccine delivery systems while improving patient compliance by reducing the need for prime-boost immunization regimen.

In this regard, degradable polymers are promising candidates for adjuvants and directed delivery vehicles because their properties can be tailored to enhance the immune response^{3–5}. While a number of degradable polymer families (polyesters⁴, polyethers⁶, and polyphosphazenes⁷) have been investigated as vaccine adjuvants, amphiphilic polyanhydrides offer a unique set of properties that allow them to function similarly to traditional adjuvants but without the challenges associated with them (i.e., protein instability, low pH environments, poor control over release kinetics, multiple dose immunization regimens, inclusion of surfactants, stabilizers, etc.)⁸. We



have previously demonstrated that amphiphilic polyanhydride particles release stable proteins in a controlled manner^{9,10} while activating APCs and inducing long-lived protective immunity in the absence of additional excipients^{5,11–13}.

Many chemistry-dependent interactions associated with materials design have been evaluated, including protein stabilization, protein release, and immune activation^{3,9–11,13}, yet little work to date has investigated the specific material properties responsible for these attributes. The identification of these properties can lead to the rational design of new and safe adjuvants that induce strong immune responses reminiscent of natural infections. Fig. 1 depicts our hypothesis that amphiphilic polyanhydride nanoparticles behave in a manner that mimics the ability of pathogens to induce a robust immune response. To test this hypothesis, we devised a transdisciplinary approach that combines polymer chemistry, cell biology, immunology, and informatics analysis to identify the properties of polyanhydrides that mimic microbial PAMPs. This approach presents a *direct* comparison between polyanhydride nanoparticles and pathogens (i.e., *Escherichia coli* and *Yersinia pestis*) based on their persistence within and ability to activate APCs. We analyzed the ability of the nanoparticles to activate APCs in comparison to lipopolysaccharide (LPS), a PAMP found on the outer membrane of Gram-negative bacteria¹⁴. Using informatics analysis to evaluate the interactions of APCs with polyanhydride nanoparticles (Fig. 1 left) or pathogens (Fig. 1 right), we have identified molecular attributes that confer “pathogen-like” behavior to amphiphilic polyanhydrides. These insights were used to design and test a single dose nanoparticle vaccine for pneumonic plague that induced long-lived protection against a lethal challenge.

Results

In order to investigate internalization and persistence of nanoparticles within APC, bone marrow-derived dendritic cells (DCs) were

incubated with fluorescently labeled polystyrene (PS) beads, 50:50 CPH:SA or 50:50 CPTEG:CPH polyanhydride nanoparticles, *E. coli* or *Y. pestis*. The phagocytic capacity of DCs was assessed using 200 nm and 2 μm PS beads as an inert control. Morphometric analysis was utilized to assess particle/bacteria count as a function of size to determine if the particles/bacteria changed in size (i.e., due to aggregation or degradation) internalization. These morphometric patterns are observed during bacterial internalization, replication and intracellular persistence and can therefore be utilized as a comparison parameter to assess the pathogen mimicking characteristics of nanoparticles. After 2 h, morphometric analysis revealed that the different particle chemistries and the bacteria were similarly internalized by DCs with respect to particle size and the number of particles (Fig. 2a and 2b). Microscopic observations at both 2 and 48 h (Fig. 3a) indicated that the majority of particles were located within late endosomes. These results are consistent with phagosome-lysosome fusion events that are part of the exogenous pathway of antigen uptake, processing, and presentation¹⁶.

The intracellular fate of pathogens (i.e., persistence, replication, rapid degradation) influences the functional capacity of APCs. In order to model the differential responses of the DCs induced by the various treatments, we characterized the intracellular fate of PS beads and polyanhydride nanoparticles, particularly in comparison to that of bacteria. At 48 h, DCs incubated with PS beads contained large numbers of individual (i.e., non-aggregating) particles (Fig. 3a). In contrast, both 50:50 CPH:SA and 50:50 CPTEG:CPH nanoparticles were found to be aggregated within lysosomes. More 50:50 CPTEG:CPH nanoparticles persisted within this intracellular compartment at 48 h (as evidenced by similar size versus number relationships) as compared to 50:50 CPH:SA nanoparticles (Fig. 3b). Because both polyanhydride nanoparticle formulations degraded, our hypothesis is that the amphiphilic 50:50 CPTEG:CPH nanoparticles maintained their size because their degradation was balanced

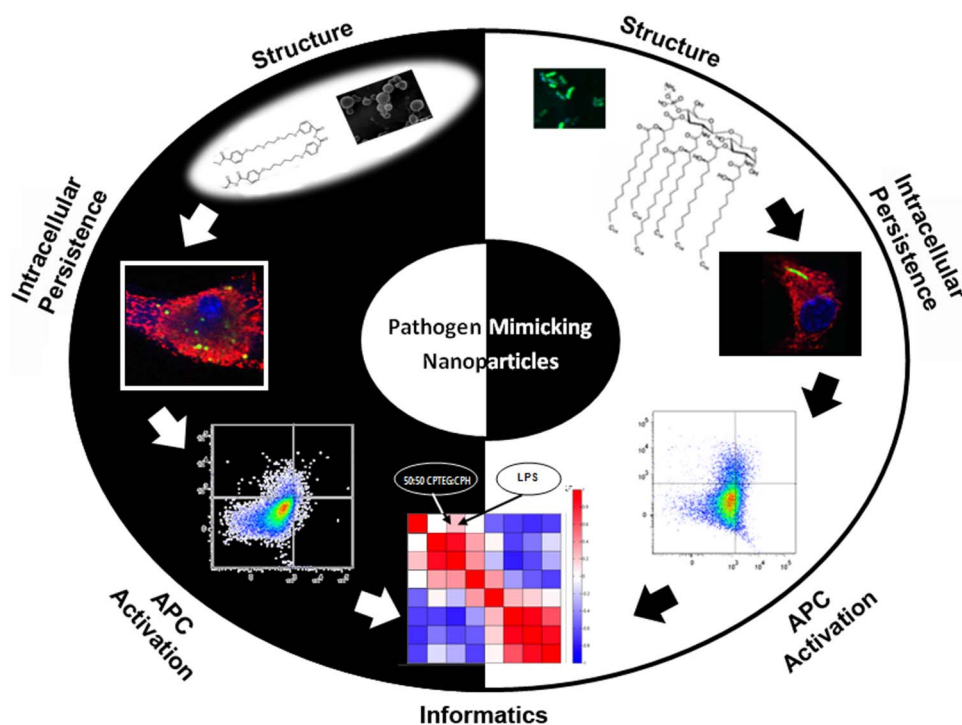


Figure 1 | Cartoon representation of the hypothesis that polyanhydride nanoparticles mimic the immune activation capacity of pathogens. Molecular structures dictate how materials are internalized and processed by APCs, which in turn leads to cellular activation. Utilizing informatics analysis, large data sets can be mined to determine which nanoparticle chemistries (left) are able to best mimic pathogen-induced activation of APCs (right). Further analysis with this approach allows for the identification of molecular descriptors that are specifically responsible for APC activation. Structures of 50:50 CPTEG:CPH (top left quadrant) and LPS (top right quadrant) were obtained as permitted¹⁵.

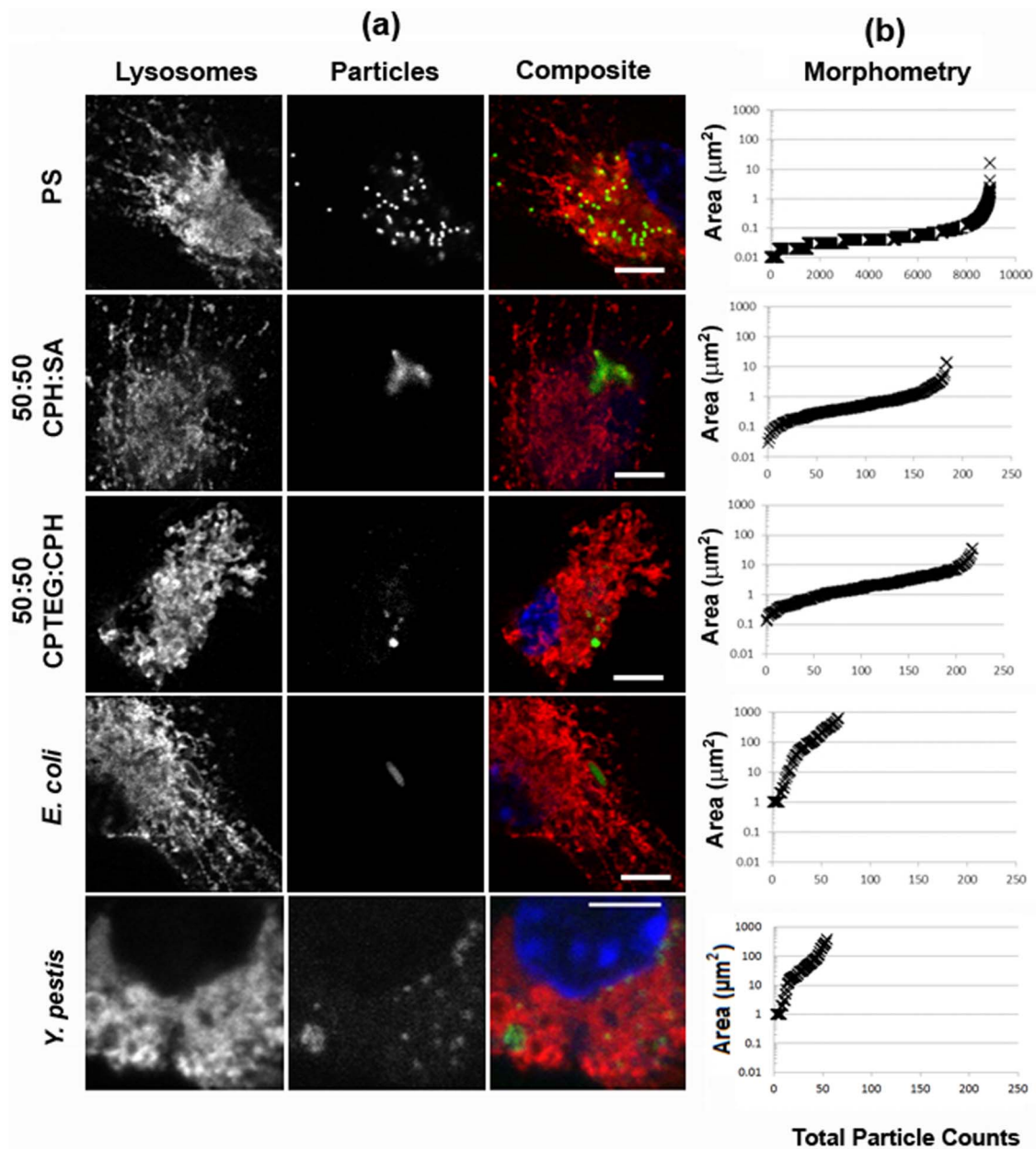


Figure 2 | Internalization of nanoparticles and bacteria (*E. coli* or *Y. pestis*) in dendritic cells at 2 h post-incubation. (a) Mouse DCs were incubated separately with either fluorescently-labeled (green) PS beads (200 nm and 2.0 µm), 50:50 CPH:SA nanoparticles, 50:50 CPTEG:CPH nanoparticles, *E. coli*, or *Y. pestis*. Fluorescent detection of phalloidin⁺ polymerized actin is shown in red. Photomicrographs shown are representative of results obtained from five independent experiments (scale bars = 5 µm). (b) Results were compiled from a morphometric analysis performed on replicate, epifluorescent photomicrographs for each treatment group. For the morphometric analysis at 2 h, data points were ordered based on size (µm²) from smallest to largest along the vertical axis and the total number of particles measured is reflected on the horizontal axis.

by their aggregation, whereas the intracellular aggregation of the hydrophobic 50:50 CPH:SA nanoparticles far exceeds their degradation (Fig. 3b).

When phagocytic cells like DCs internalize pathogens, they create intracellular particulate agglomerates after 48 h as observed in Fig. 3. When compared to *E. coli* and *Y. pestis* internalization (Figs. 2 and 3), the amphiphilic 50:50 CPTEG:CPH nanoparticles appeared to be similarly internalized and persisted at 2 and 48 h, respectively. This persistence of the 50:50 CPTEG:CPH nanoparticles suggests that the stimuli provided by these particles to activate DCs would not rapidly wane, much like that provided by persisting and replicating bacteria¹⁷. Indeed, comparing morphometric results between intracellular bacteria and internalized 50:50 CPTEG:CPH nanoparticles (Fig. 3b) revealed similar persistence patterns. In contrast,

hydrophobic 50:50 CPH:SA nanoparticles aggregated over time, resulting in larger but fewer particles.

Furthermore, lysosomal localization of the 50:50 CPTEG:CPH nanoparticles provides an optimal location for antigen presentation with concomitant cell activation^{18,19}. It is of note that while the majority of the nanoparticles were found within Lamp1⁺ lysosomes at 48 h, a minor portion of intracellular particles were not, and could be either within Lamp1⁻ vesicles or, more likely, free within the cytosol. The capacity for antigen release within both lysosomal and cytosolic compartments could enable cross presentation and enhanced antigen presentation – experiments to test this hypothesis are currently underway in our laboratories.

Given the central role of DCs in the development of robust immune responses, we next assessed the capacity of the polyanhydride

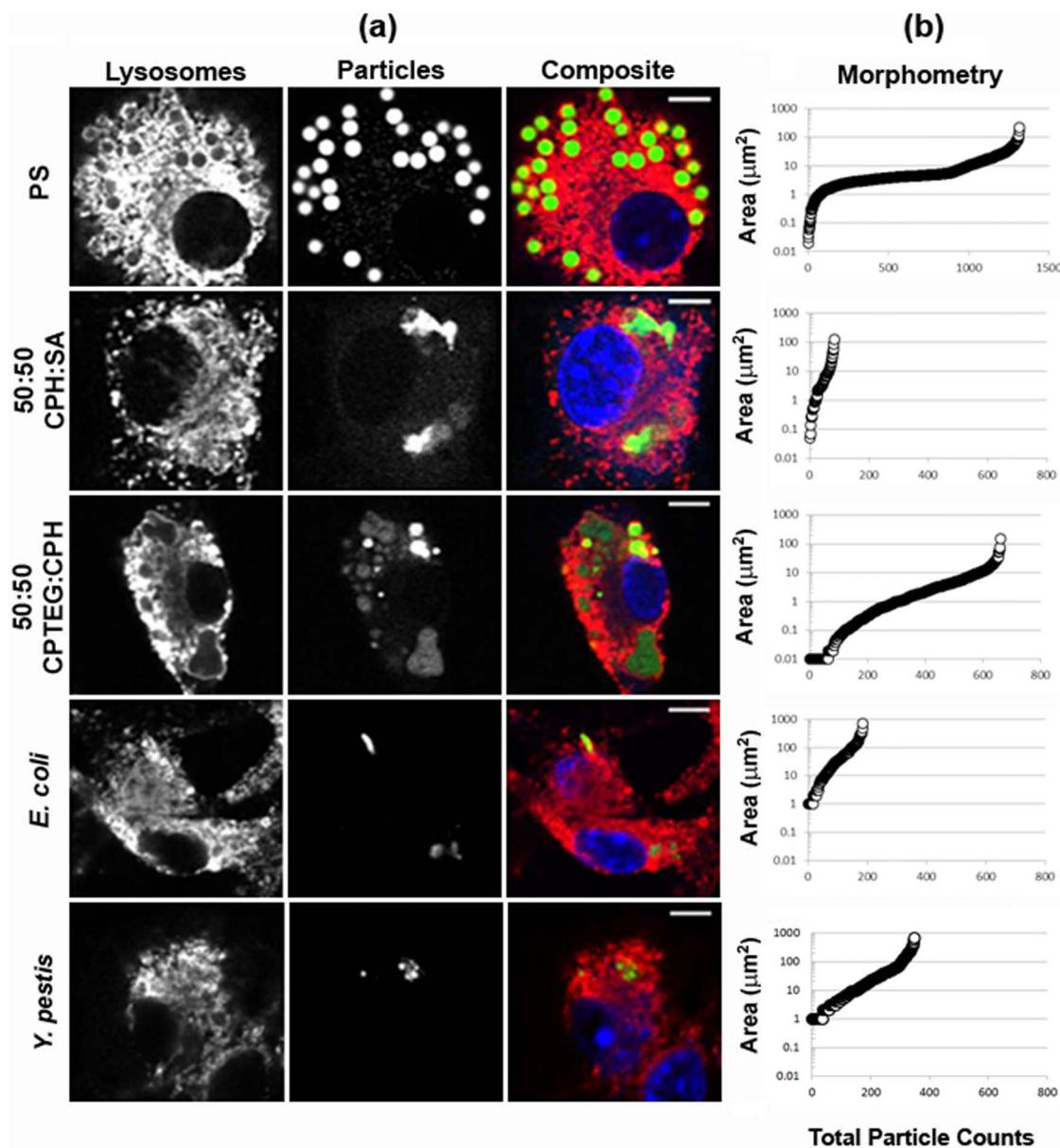


Figure 3 | Persistence of amphiphilic 50:50 CPTEG:CPH nanoparticles and bacteria (*E. coli* or *Y. pestis*) in dendritic cells at 48 h post-incubation. (a) Fluorescent antibody detection of LAMP-1⁺ lysosomes is shown in red. Photomicrographs shown are representative of results obtained from five independent experiments (scale bars = 5 µm). (b) See caption for Figure 2b).

nanoparticles to activate DCs in comparison to LPS. DCs stimulated with hydrophobic 50:50 CPH:SA nanoparticles failed to increase surface expression of major histocompatibility complexes (MHC) I and II and the T cell co-stimulatory molecules CD86 and CD40 over non-stimulated DCs (Fig. 4a). In contrast, stimulation of DCs with amphiphilic 50:50 CPTEG:CPH nanoparticles resulted in a significant increase in all these activation markers over the non-stimulated controls (Fig. 4a). Moreover, 50:50 CPTEG:CPH nanoparticles activated DCs to a similar degree as did treatment with LPS in terms of MHC I, MHC II, and CD86 expression but not CD40 expression (Fig. 4a). Significantly greater quantities of the pro-inflammatory cytokines IL-6, IL-12p40, and TNF- α were secreted from DCs exposed to 50:50 CPH:SA or 50:50 CPTEG:CPH nanoparticles as compared to non-stimulated DCs; however, the amounts of cytokines induced by nanoparticles were far less than those induced by LPS (Fig. 4b). This data reveals a potential benefit of 50:50 CPTEG:CPH nanoparticles as a vaccine adjuvant, because excessive CD40 signaling and pro-inflammatory cytokine production can lead

to adverse consequences. Indeed, mice deficient in CD40 are more resistant to the effects of polymicrobial sepsis, produce less IL-6 and IL-12, and sustain less systemic organ damage than wild-type controls²⁰. Together, our data demonstrate that amphiphilic 50:50 CPTEG:CPH nanoparticles possess pathogen-like characteristics that promote DC activation in a tempered context that would likely limit the development of an overwhelming inflammatory response, thus making them excellent vaccine delivery vehicles.

We then investigated the relationships between various polyanhydride chemistries (represented by CPH:SA and CPTEG:CPH copolymers), *E. coli*, *Y. pestis*, and LPS for intracellular persistence and DC activation using a “blinded” approach to informatics analysis (Fig. 5). Specifically, the trends in these relationships were quantitatively modeled based on a set of molecular descriptors chosen to interrogate pathogen-like behavior. The library of descriptors was built based partially on the structural descriptors²¹ of CPH:SA and CPTEG:CPH copolymers and microbial components (i.e., LPS) and on experimentally measured descriptors such as glass transition temperature,

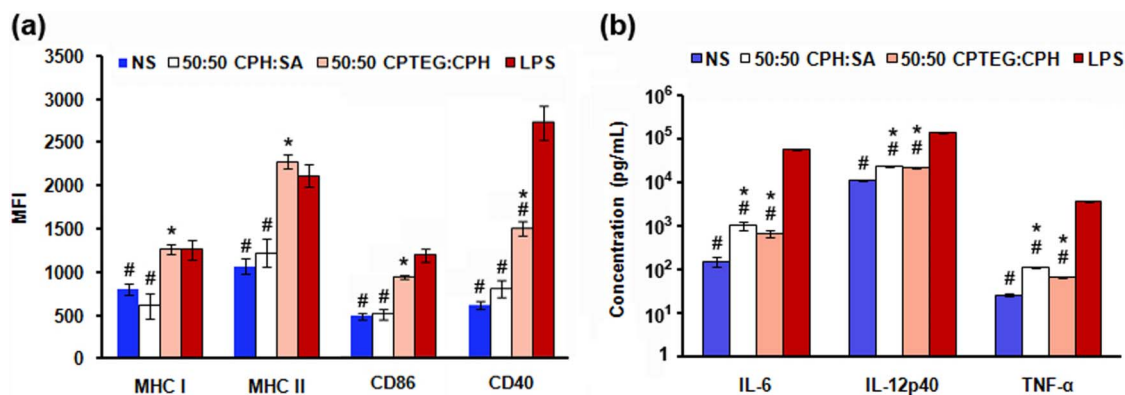


Figure 4 | Polyanhydride nanoparticles generate an activated DC phenotype similar to that of DCs stimulated with LPS. Cell surface marker expression (a) and cytokine secretion (b) for non-stimulated (NS, negative control) DCs, DCs stimulated with LPS (positive control) or nanoparticles composed of either 50:50 CPH:SA or 50:50 CPTEG:CPH for 48 h. A minimum of eight replicates was used per group. Error bars represent standard error. MFI = mean fluorescence intensity. * indicates $p \leq 0.002$ compared to NS and # indicates $p \leq 0.002$ compared to LPS.

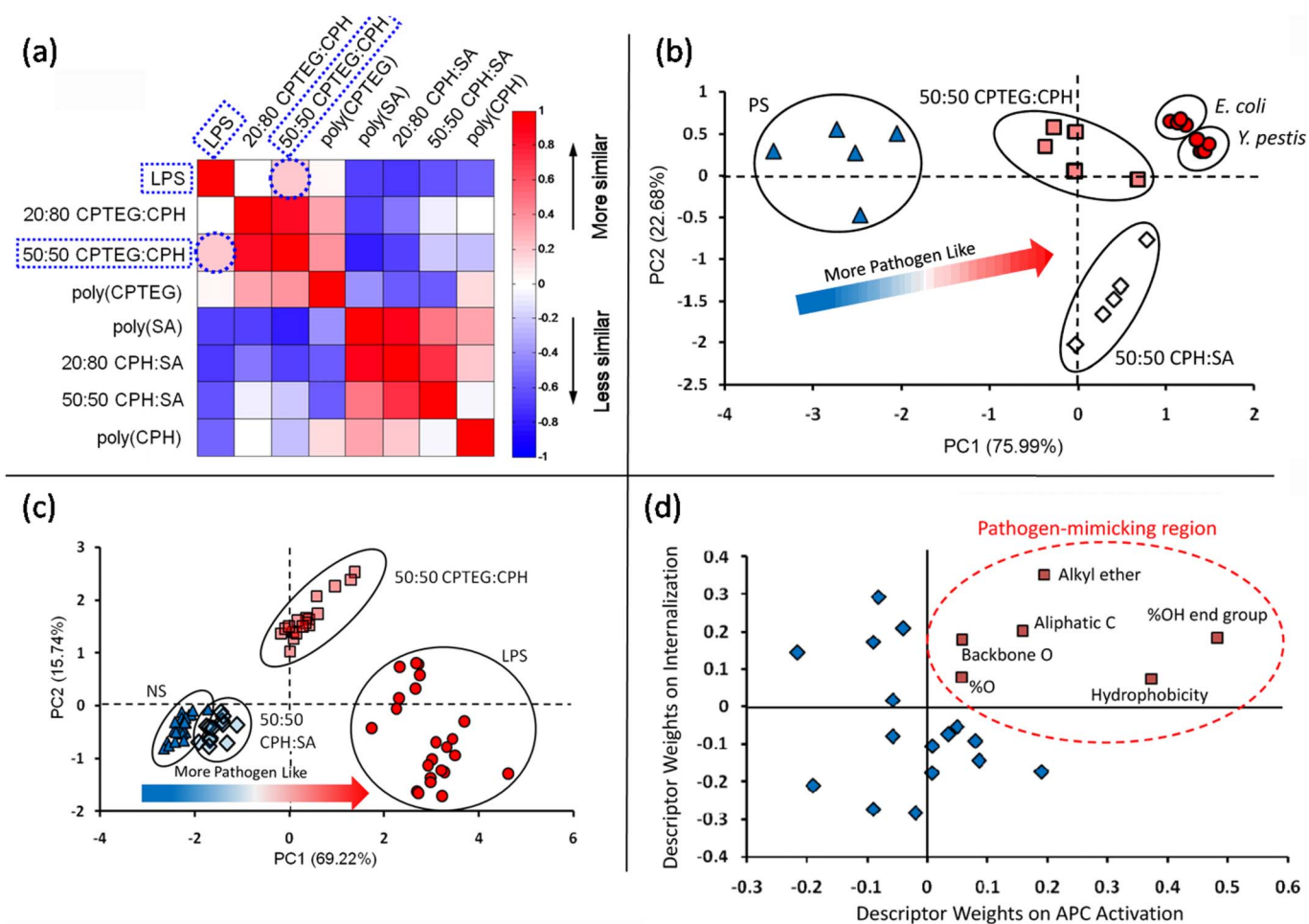


Figure 5 | Informatics analysis using specific molecular descriptors to identify polymer chemistries that behave in a pathogen-like manner.

(a) Similarity (correlation) matrix for the paired comparison of LPS and various polyanhydride copolymers in terms of molecular descriptors; the red color indicates highest similarity and blue represents lowest similarity with LPS. Based on Pearson's correlation, 50:50 CPTEG:CPH showed the highest similarity with LPS (indicated by the blue dotted circles). (b) In terms of intracellular persistence, 50:50 CPTEG:CPH nanoparticles are more pathogen-like than 50:50 CPH:SA or PS. (c) In terms of DC activation, 50:50 CPTEG:CPH nanoparticles are more pathogen-like than 50:50 CPH:SA or NS. (d) Using a PLS model linking molecular descriptors with the positions of the nanoparticle formulations in panel (b) representing the y-axis and panel (c) representing the x-axis, the descriptors that influence pathogen-like behavior in terms of both nanoparticle persistence and DC activation were identified (relevant descriptors labeled on figure).


Table 1 | Molecular descriptors (1–23) defined by Bicerano²¹ and material property measurements (24–28) for the enumeration of polymer and LPS features

ID	Molecular descriptor	Description
1	$^0\chi$	Atomic index 1
2	$^0\chi^v$	Atomic index 2
3	$^1\chi$	Connectivity index 1
4	$^1\chi^v$	Connectivity index 2
5	<i>BB_index1</i>	Backbone index 1
6	<i>BB_index2</i>	Backbone index 2
7	<i>SG_index</i>	Backbone index 3
8	<i>N_SP</i>	Number of atoms in the shortest path across the backbone of a polymeric repeat unit
9	<i>N_C</i>	Number of carbon atoms
10	<i>N_O</i>	Number of oxygen atoms
11	<i>N_H</i>	Number of hydrogen atoms
12	<i>N_ester</i>	Number of backbone -COO-
13	<i>N_ether</i>	Number of -O- in a polymeric repeat unit
14	<i>N_aromatic_ring</i>	Number of aromatic rings in a polymeric repeat unit
15	<i>N_CH₂</i>	Number of CH ₂ groups in a polymeric repeat unit
16	<i>N_alkyl_ether</i>	Number of ether (R-O-R') linkages between two units R and R' both of which are connected to the alkyl carbon atom
17	<i>N_nh</i>	Total number of non-hydrogen atoms in a repeat unit
18	<i>N_mv</i>	$2*N_{ester} + 3*N_{ether}$
19	<i>N_K</i>	$-3*(N_{ether})^{-3}*N_{acrylic_ester}$
20	<i>N_rot</i>	Total number of rotational degrees of freedom parameter
21	<i>N_dc</i>	$7*N_{backbone_O} + 12*N_{side\ Group_O}$
22	<i>N_group</i>	$-N_{alkyl_ether} + 7*N_{CO} + 2*N_{other\ C=O}$
23	<i>MW</i>	Molecular weight
24	<i>% OH end group</i>	Percentage of -OH groups
25	<i>Hydrophobicity</i>	Water contact angle
26	<i>% MW loss</i>	Percent loss of polymer molecular weight from NMR and GPC
27	<i>% Erosion</i>	Percent of polymer eroded, from erosion kinetics experiments
28	<i>T_g</i>	Glass transition temperature from DSC

hydrophobicity, and erosion kinetics (Table 1 and Supplementary Data Table 1). Thermal properties of LPS are known and correlate closely with those of whole bacteria^{22,23}. The chemical and structural similarities between the polyanhydride chemistries and LPS were explored using a correlation similarity metric²⁴, where similarity was defined by the proximity between chemistries in the descriptor space. Of the polymer formulations evaluated, the similarity plot shown in Fig. 5a demonstrates that the amphiphilic 50:50 CPTEG:CPH has the highest similarity with LPS.

Using principal component analysis (PCA), we identified the relationships between various polyanhydride formulations and pathogens or LPS in the context of intracellular persistence and DC activation (Fig. 5b and 5c, respectively)²⁵. An Euclidean geometrical mapping metric that considers both the distance between two points as well as the angle between point-origin-point was used to identify similarity in the attributes associated with the targeted functionality of pathogen-like behavior. For both intracellular persistence (Fig. 5b) and DC activation (Fig. 5c) data, the angular direction of correlation was drawn as an arrow and defined based on maximizing the difference between the target (i.e., LPS or bacteria) and the nanoparticle formulations. The geometrical proximity of the angular projection of the 50:50 CPTEG:CPH nanoparticle cluster to the target is closer than that of the 50:50 CPH:SA nanoparticle cluster, suggesting that the amphiphilic 50:50 CPTEG:CPH nanoparticles behave more similarly to bacteria or LPS.

In addition, we developed predictive models via partial least squares to connect experimental measurements with molecular descriptors by predicting the PCA values of Figs. 5b and 5c as a function of molecular descriptors. The combined results of the models are shown in Fig. 5d, where the horizontal axis represents DC activation and the vertical axis represents intracellular persistence. In both cases, the increasing positive value of a molecular descriptor indicates increasing pathogen-like behavior. The specific descriptors

responsible for being pathogen-like in terms of DC activation and intracellular persistence identified by our informatics methods are: i) percentage of hydroxyl end groups, ii) hydrophobicity, iii) alkyl ethers, iv) aliphatic carbons, and v) backbone oxygen moieties.

The concept of ‘similarity’ defined in terms of molecular descriptors and physicochemical properties is a valuable tool to screen appropriate adjuvant candidates, enhancing our understanding of the key molecular attributes responsible for optimal adjuvant activity as well as pathogen-like behavior. For example, we identified hydroxyl end groups as an important molecular feature of the 50:50 CPTEG:CPH nanoparticles. When this polymer degrades, it exposes numerous hydroxyl end groups. Identification of hydroxyl end groups as a pathogen-like characteristic is consistent with the structural components of LPS²⁶ and may explain the observed similarities in DC activation profiles (Fig. 4). Indeed, recent studies by Hubbell and co-workers have shown that hydroxyl end groups on nanoparticles significantly influenced complement activation and subsequent DC trafficking to lymph nodes²⁷. It is striking that descriptors such as hydroxyl end groups, alkyl ethers, and backbone oxygen moieties are commonly observed molecular constituents on pathogens and that the same parameters were identified by the (blinded) informatics analysis when complementary but dissimilar experimental data (from microscopy and flow cytometry) were compared.

Finally, we demonstrated the success of utilizing a pathogen-mimicking nanoparticle vaccine to induce long-term protection against pneumonic plague. Our previous studies have shown that encapsulation of F1-V antigen into amphiphilic polyanhydride particles preserved the antigenicity of F1-V upon release²⁸. In this work, we designed and tested three vaccine formulations based on 50 µg of soluble F1-V (S₅₀), S₅₀ adjuvanted with 500 µg of blank 50:50 CPTEG:CPH nanoparticles (S₅₀ + E₀), and 40 µg of soluble F1-V and 10 µg of encapsulated F1-V in 500 µg of 50:50 CPTEG:CPH



nanoparticles ($S_{40} + E_{10}$). C57BL/6 mice were intranasally vaccinated with these formulations and subsequently challenged at 280 days post-vaccination with 1200 CFU of *Y. pestis* CO92. In these experiments, none of the mice vaccinated with S_{50} or $S_{50} + E_0$ survived the challenge, whereas all the mice treated with the $S_{40} + E_{10}$ formulation survived (Fig. 6a). Protection from the lethal challenge was strongly correlated with the F1-V-specific antibody titer and antibody avidity. Mice vaccinated with either $S_{50} + E_0$ or $S_{40} + E_{10}$ generated significantly higher F1-V-specific antibody titers than animals vaccinated with S_{50} (Fig. 6b). However, by 6 weeks post-vaccination, anti-F1-V IgG titers began to wane in mice vaccinated with $S_{50} + E_0$. In contrast, the F1-V-specific IgG titer in $S_{40} + E_{10}$ vaccinated mice was sustained for 275 days. In addition to antibody titer, we evaluated avidity as a measure of the quality of the antigen-specific antibody. As shown in Fig. 6c, mice vaccinated with either

S_{50} or $S_{50} + E_0$ formulations developed a low avidity F1-V-specific IgG response. In contrast, mice vaccinated with $S_{40} + E_{10}$ generated a higher avidity anti-F1-V specific IgG antibody by 3 weeks post-vaccination that was sustained for 275 days. Together, the data in Fig. 6 demonstrate that the pathogen-mimicking nanoparticle vaccine successfully protected animals against a lethal *Y. pestis* challenge 280 days after a single intranasal administration.

The ability to induce high titer antibody responses to F1-V following intranasal vaccination has been shown to be important for the protection of mice against pneumonic *Y. pestis* infections^{29–31}. Each of these studies demonstrated the ability to induce high titer antibody responses to F1 and/or V; however, high titer antibody responses did not always correlate with protection. This suggests that the quality (i.e., avidity) of the antibody response may also be a critical factor in a protective immune response. In this regard, the studies reported herein have demonstrated that the use of a polyanhydride nanoparticle-based vaccine induced long-lived, high titer, and high avidity anti-F1-V antibody responses following a single intranasal vaccination. Unlike the previous studies, these responses were induced without the use of alum or TLR ligands. Similar to that induced by a natural infection, the robustness of the immune response induced by a single administration of the nanoparticles was remarkable in that complete protection of mice from *Y. pestis*-induced pneumonia was observed for at least 280 days post-vaccination.

Discussion

An ideal vaccine delivery system should consist of non-toxic materials that mimic the immune enhancing capabilities of PAMPs and activate APCs without deleterious side effects. Our results demonstrate that by combining cell population-based and individual cell-based analyses with informatics, the molecular properties responsible for the “pathogen-mimicking” behavior of amphiphilic nanoparticles can be identified. The ability to change size and shape as a result of interacting with the cell, without being eliminated, was best exhibited by the 50:50 CPTEG:CPH nanoparticles. This observation can be equated to the intracellular persistence and survival of many microbial pathogens including *Brucella*, *Salmonella*, and *Mycobacterium* species³². Moreover, the 50:50 CPTEG:CPH nanoparticles possessed the ability to enhance DC activation similar to LPS without inducing a toxic cytokine storm. The informatics analysis confirmed the similarity between 50:50 CPTEG:CPH nanoparticles and bacteria/LPS and identified the molecular descriptors responsible for their similarity in terms of intracellular persistence and DC activation. Vaccination of mice with 50:50 CPTEG:CPH nanoparticles administered as a single intranasal dose induced high-titer and high-avidity antibody responses and 100% protection against a live *Y. pestis* challenge for at least 280 days post-vaccination.

Our transdisciplinary approach to adjuvant design provides a toolbox that can be used to rationally identify and design “pathogen-mimicking” materials for use in combination vaccines that will induce the appropriate immune modulation to protect against a wide range of pathogens. We hypothesize that the insights gained by our approach will elucidate important principles of rational design of adjuvants. We foresee that the immunological and public health challenges associated with the control of emerging and re-emerging infectious diseases may not be met by the use of off-the-shelf adjuvants and delivery systems that must be supplemented with TLR ligands or excipients and administered multiple times to obtain protection³³. Instead, these challenges will likely require the development of novel vaccine delivery platforms that appropriately modulate the immune response and improve patient compliance by requiring only a single administration.

Methods

Materials. Carboxylic diacid monomer synthesis required the use of 1, 6-dibromohexane (98.5%), 4-hydroxybenzoic acid (96%), 1-methyl-2-pyrrolidinone anhydrous (99.5%), triethylene glycol (99%) purchased from Sigma Aldrich

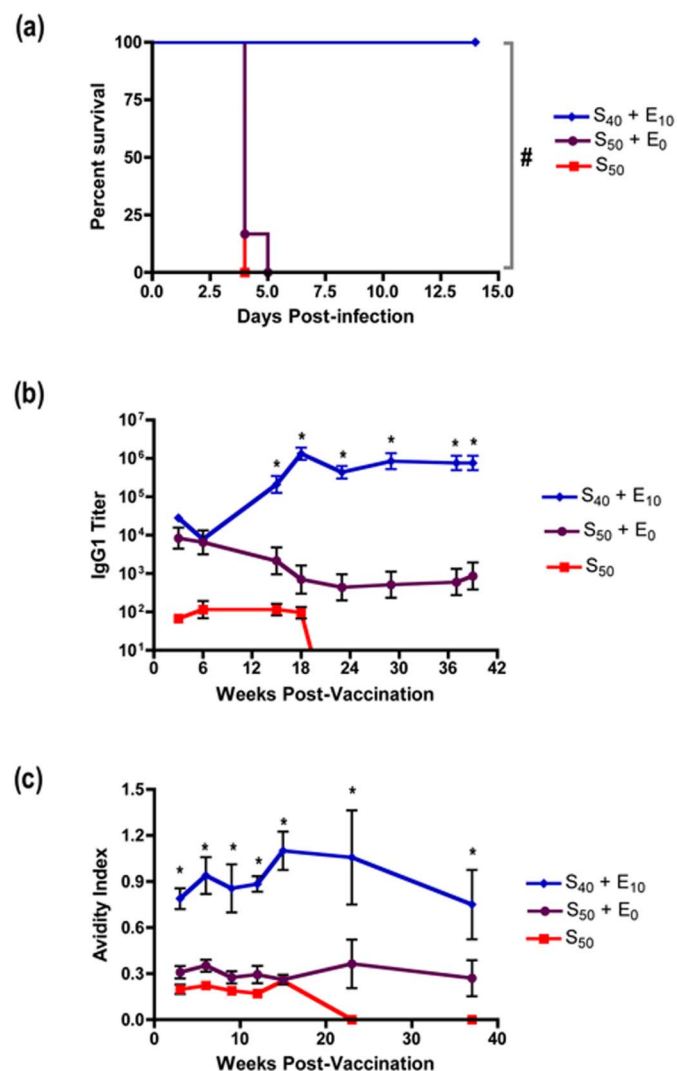


Figure 6 | Single administration of 50:50 CPTEG:CPH nanoparticle vaccine provided complete protection against lethal *Y. pestis* challenge and induced a long-lived, high-titer, high-avidity antibody response. (a) C57BL/6 mice were challenged intranasally with 1200 CFU (LD100) *Y. pestis* CO92 at 280 days post intranasal vaccination ($n = 5–6$ per group). (b) Kinetics of IgG antibody titer throughout 275 days post-vaccination ($n = 6$ per group). (c) Kinetics of IgG antibody avidity throughout 275 days post vaccination ($n = 6$ per group). Titer and avidity data are presented as mean \pm SEM. For (a), # represents a statistically significant difference from all other treatments at $p \leq 0.0016$ and for (b) and (c) * represents a statistically significant difference from all other treatments at $p \leq 0.05$.



(St. Louis, MO); 4-*p*-fluorobenzonitrile (98%) purchased from Apollo Scientific (Stockport, Cheshire, England); and sodium hydroxide, sulfuric acid, acetonitrile, dimethyl formamide, toluene and potassium carbonate purchased from Fisher Scientific (Fairlawn, NJ). Acetic anhydride, chloroform, petroleum ether, ethyl ether, methylene chloride and hexanes were purchased from Fisher Scientific. DC culture medium included RPMI 1640, HEPES buffer, L-glutamate, penicillin-streptomycin, gentamycin acquired from Mediatech (Herndon, VA); heat inactivated fetal calf serum acquired from Atlanta Biologicals (Atlanta, GA); and GM-CSF acquired from PeproTech (Rocky Hill, NJ). Materials used in flow cytometry included fixative buffer (BD Bioscience); mouse serum; anti-mouse CD16/32 FcγR purchased from eBioscience (San Diego, CA); β-mercaptoethanol and unlabeled rat immunoglobulin purchased from Sigma Aldrich; *E. coli* lipopolysaccharide (LPS) was acquired from Sigma Aldrich. FluoSpheres® (200 nm and 2 μm) were purchased from Invitrogen (Carlsbad, CA). *Y. pestis*, strain CO92 (NR-641) and the *Y. pestis* F1-V fusion protein, recombinant from *E. coli* (NR-4525), were obtained from the Biodefense and Emerging Infections Repository (Manassas, VA).

Polymer Synthesis, Nanoparticle Fabrication, and Characterization. Sebacic acid was purchased from Sigma Aldrich. Synthesis of CPTEG and CPH diacids, SA and CPH pre-polymers, and CPH:SA and CPTEG:CPH copolymers was performed as previously described^{34,35}. The polymers were characterized using ¹H nuclear magnetic resonance spectroscopy, gel permeation chromatography, and differential scanning calorimetry. All properties evaluated of the synthesized polymers were within accepted ranges^{34,35}.

Both FITC-dextran loaded and blank nanoparticles were fabricated using the protocol reported in Ulery et al.¹³. The endotoxin levels of these particles were tested using a *Limulus* Amebocyte Lysate (LAL) QCL-1000 test kit (Cambrex, Walkersville, MD) as described previously¹². The results indicated that the particles exhibited an endotoxin content of less than 0.1 EU/mL, which is five times lower than the maximum level permitted by the United States FDA for new drugs tested by the LAL test³⁶. Nanoparticle morphology and size was investigated using scanning electron microscopy (JEOL 840A, JEOL Ltd., Tokyo, Japan) and appeared to be spherical in shape. The size of the 50:50 CPTEG:CPH nanoparticles was 206 ± 47 nm and that of the 50:50 CPH:SA nanoparticles was 415 ± 76 nm (data presented as mean ± standard deviation from three independent batches of nanoparticles). No differences in size or morphology were found for blank or 1% FITC-dextran-loaded nanoparticles across all chemistries.

Culture, Stimulation, and Analysis of C57BL/6 Dendritic Cells. All experiments involving animals were carried out in accordance with procedures approved by the Iowa State University Institutional Animal Care and Use Committee. The dendritic cells (DCs) were prepared from bone marrow cells isolated from the femurs, tibias, humeri and ilia of C57BL/6 mice (obtained from Harlan Sprague Dawley). DCs were cultured, stimulated and assessed for cell surface marker expression and cytokine secretion as described previously with the same materials¹¹. Nanoparticle stimulation doses of 125 μg/mL and LPS stimulation doses of 200 ng/mL were utilized.

Intracellular Localization. Intracellular localization of nanoparticles was compared between PS beads at a 1:1000 dilution and 250 μg/mL of 1% FITC-dextran loaded nanoparticles composed of either 50:50 CPH:SA or 50:50 CPTEG:CPH. DCs were infected with green fluorescent protein (GFP)-expressing *E. coli* HB101 or *Y. pestis* Δpgm at multiplicity of infection (MOI) of 100 and 10, respectively. Following nine days of culture, DCs were pulsed with particles for 30 min (2 h sample) or 6 h (48 h sample). *E. coli* and *Y. pestis* were incubated for 30 min and 48 h with DCs. Cells were subjected to immunofluorescence microscopy to observe time-dependent intracellular trafficking of individual nanoparticles within DCs, similarly to previous work¹³. Polymerized actin was labeled with Alexa 568 conjugated phalloidin mushroom toxin (Molecular Probes-Invitrogen) and lysosomes were stained with ID-4B anti-LAMP-1 (lysosomal-associated membrane protein 1). Epifluorescence and immunofluorescence microscopy were performed using an inverted Olympus Fluoview™ 1000 laser scanning confocal microscopy. Intracellular trafficking analysis and final images were prepared using ImageJ v1.36b (NIH, Bethesda, MD) loaded with particle counting algorithms.

Vaccinations and Challenge of Mice. *Y. pestis* CO92 was grown overnight at 37°C in heart-infusion broth supplemented with 0.2% D-galactose. Eight-week-old female C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained under SPF conditions. The Institutional Committees on Animal Care and Use at either Iowa State University or Albany Medical College approved all procedures involving animals. Mice were vaccinated and challenged and the methods to measure antibody titer and avidity were performed as described previously⁵.

Data and Statistical Analysis. JMP software (SAS Institute, Cary, NC) was used to make comparisons between treatments and the negative (NS) control and the positive (LPS) control using the student T-test.

Informatics Analysis. The similarity matrix shown in Fig. 5a was constructed with the correlation (R) metric defined by the following equation:

$$R(x,y) = \frac{\text{cov}(x,y)}{\sigma_x \sigma_y} = \frac{\sum_{i=1}^n (x_i - x_{\text{mean}})(y_i - y_{\text{mean}})}{\sqrt{\sum_{i=1}^n (x_i - x_{\text{mean}})^2} \sqrt{\sum_{i=1}^n (y_i - y_{\text{mean}})^2}}$$

Here, *x* and *y* refer to a block of chemistries (LPS, 20:80 CPTEG:CPH, 50:50 CPTEG:CPH, 20:80 CPH:SA, 50:50 CPH:SA, poly(CPTEG), poly(CPH), and poly(SA)), *cov*(*x*,*y*) is the covariance between *x* chemistry and *y* chemistry, and σ is the standard deviation. Note that only molecular descriptors 1–25 and 28 from Table 1 were used to generate this similarity matrix.

The PCA analysis for nanoparticle persistence (Fig. 5b) used count, total area, and average size from epifluorescence microscopy data, while that for DC activation (Fig. 5c) used MHC I, MHC II, CD40, CD86, IL-6, IL-12p40, and TNF-α data. PLS regression connected the descriptor data set with the results of the PC analysis. By projecting the data onto a high-dimensional hyperplane defined by PCA analysis of training data, the impact of the descriptors on the property were identified, while taking co-linearity into account³⁷.

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Author contributions

B.D.U. and L.K.P. designed the overall scope of the work and performed the flow cytometric studies and wrote the paper. Y.P. performed the microscopy experiments. C.-S.K. and S.R.B. performed the informatics analysis. B.C.-C., B.D.U., and Y.P. performed the in vivo immune response experiments. D.K. performed the challenge studies. A.E.R., B.H.B., K.R., M.J.W., D.W.M., and B.N. contributed to study design, data analysis and interpretation, and manuscript preparation.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

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